

AÇÕES ANTI - INFLAMATÓRIA E ANTINOCICEPTIVA DO EXTRATO ETANÓLICO DE *Hymenaea courbaril* L. EM ROEDORES
(ANTI - INFLAMMATORY AND ANTINOCICEPTIVE ACTIONS OF ETANOLIC EXTRACT FROM *Hymenaea courbaril* L. IN RODENTS)

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ABSTRACT

The anti-inflammatory and antinociceptive actions of the Etanolic Extract from *Hymenaea courbaril* L. (EEHC) were evaluated for the first time in animal models. The extract was produced using the stem bark. The preparation was administered to rodents *per oral*. EEHC has efficiently reduced the abdominal contortions induced by acetic acid; time of paw licking in formalin test; paw edema induced by carrageenan and dextran; leukocyte migration after intraperitoneal injection of carrageenan and myeloperoxidase expression on peritoneal exsudate. EEHC also improved the histopathological damages in the rat paw after carrageenan inflammation as well as reduced TNF- α expression in the same tissue. Doses varying from 1 to 400 mg/kg were tested. The dose of 100 mg/kg presented significant results in all experiments ($p < 0.05$). The data suggest intense anti-inflammatory and antinociceptive effects associated to extract from *Hymenaea courbaril*. Further studies must be carried out to elucidate all mechanisms involved.

KEYWORDS: *Hymenaea courbaril*, Antinociceptive, Carrageenan, Inflammation, Jatobá, TNF- α .

INTRODUCTION

Hymenaea courbaril L., *Leguminosae*, popularly known as Jatobá, is a tree randomly found in northeast Brazil. It is a great port tree that could reach 40 meters high depending on the sub specie. The plant does not lose its leaves during the season variation and could grow in not well fertilized soil but with a good drainage (Matos, 2002).

Etnobotanic literature describes the use of jatobá as a powder in culinary preparations as well as the use of steam bark in the form of a decoction 10% to heal wounds and to treat cough. In fact, syrup with *H. courbaril* was produced in the early 20th century but the lack of information and the development of international pharmaceutical companies have put this preparation aside. Jatobá presents also in popular medicine a well-accepted anti-inflammatory action and it is also used during recoveries after extenuating diseases (Matos, 2002). Extract from jatobá bark are widely used in Amazonia to overcome fatigue and as a nutritional supplement (Nogueira *et al.*, 2001; Abdel-Kader *et al.*, 2002).

The plant phytochemical analysis has demonstrated the presence of essential oil in all its parts including the steam bark. It contains sequiterpene, diterpenes, cardienes, carioflene,

copalic acid, copaenes and other analogs. Among its fixed components it could be cited epicatechine and isoenantiocomunic acids (Matos, 2002).

It was reported the presence of many bioactive components in Jatobá such as terpenoids and oligomeric sugars and therefore to these compounds were related biological activities (Imamura *et al.*, 2004; Lima-Nishimura *et al.* 2003). A recent study has successfully determined the chromatography profile of stem bark extract from *Hymenaea courbaril*. It also revealed the presence of large amount of procyanidins which demonstrates strong antioxidative and anti-inflammatory properties (Sasaki *et al.*, 2009).

The popular use of *Hymenaea courbaril* L. justifies the development of this study. For the first time an article reveal the preclinical properties of jatobá in models of inflammation in rodents. The aim of the present study was to make a screening of *H. courbaril* pharmacological actions and to bring grounded data to its usage.

METHODOLOGY

Plant material

Samples from *H. courbaril* were collected in central region of the state of Ceará, Brazil. A sample was deposited at the herbarium Prisco Bezerra at Federal University of Ceará under the number 0999.

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Preparation of the extracts

Stem bark material from *H. Courbaril* (700 g) was dried, triturated and the process of extraction by maceration was undertaken with cold ethanol. After partial concentration (under vacuum) 55 g of extract were obtained. The preparation showed the following features: red-brown color, a strong wood scent and paste consistence.

Animals

Male Swiss mice (20–25 g) and male Wistar rats (200–250 g) were from the Central Animal House of the Federal University of Ceará, Brazil. The animals were housed at $24 \pm 2^\circ\text{C}$, under a 12-h light/12-h dark cycle, and had free access to a standard pellet diet (Purina chow) and tap water. They were deprived of food for 8 h before the experiments, except for drinking water. The animals were treated in accordance to the current law and the NIH Guide for the Care and Use of Laboratory Animals. The project was previously approved by the Animal's Ethics Committee of the Faculty of Medicine of the Federal University of Ceará. The study was approved by the ethics committee with the 13/2014 protocol

Abdominal contortions induced by acetic acid

Mice in groups of 8 each were treated with EEHC (100, 200 and 400 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.) or vehicle (distilled water). One hour later, each animal received a intraperitoneal injection of acetic acid (0,6%, 0,2 mL/10 g, i.p.). The number of abdominal contortions was recorded during 30 min to each animal.

Nociception induced by formalin

The mice formalin test is a model for tonic pain and localized inflammatory pain. The formalin test presents two phases of responses, the first one (0-5 min) indicates neurogenic nociception, and the second one (15-30 min) indicates inflammatory nociception. Mice in groups of 8 each were treated with EEHC (100, 200 and 400 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.) or vehicle (distilled water). Thirty minutes later, each animal received a subcutaneous injection in the right paw of 20 μL formalin (1%). The time each mouse spent licking the injected paw was recorded in seconds.

Paw edema induced by carrageenan

Groups of 8 mice each were treated with EEHC (1, 10 and 100 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.), or vehicle (distilled water, p.o.). One hour after treatment, each animal received a

subcutaneous application in the right paw of 0.02 mL of carrageenan (1%). The left paw received the same volume of saline. The edema was measured as the difference between the paws (for the doses of 1, 10 and 100 mg/kg), and was recorded after one, two, three, four and twenty-four hours after the carrageenan administration. The edema was expressed in milliliter and assessed by a plethysmometer from Ugo Basile, Italy. Also the edema was recorded as the difference of weight between the injected and the non-injected paw at the 4th hour after carrageenan application (for the doses of 1, 10 and 100 mg/kg).

Paw edema induced by dextran

Groups of rats (n = 8) were treated with EEHC (1, 10 and 100 mg/kg, p.o.), ciproheptadin (10 mg/kg, p.o.), or vehicle (distilled water, p.o.). One hour after treatment, each animal received a subcutaneous injection in the right paw of 0.02 mL dextran (1%). The left paw received the same volume of saline. The edema was measured as the difference between the paws and was recorded after half, one, one and a half, and two hours after the dextran administration. The edema was expressed in milliliters, and was assessed by a plethysmometer (Ugo Basile, Italy).

Peritonitis induced by carrageenan

Rats were treated with EEHC (100 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.) or vehicle (distilled water, p.o.). One hour after treatment, each animal received an intraperitoneal injection of 1 mL of carrageenan (700 μg / per animal) to induce the inflammatory process. Four hours later, the animals were sacrificed. The peritoneal cavity was washed with 10 mL phosphate buffered saline (PBS) containing heparin, and the exsudate was then collected for analysis. An exsudate sample of 0.02 mL was taken, and a 0.4 mL TURK solution was added to that. Then, the total number of leukocytes was counted in a Neubauer chamber, using an optic microscope. The results were expressed as means (x $10^3/\text{mm}^3$) of the number of leukocytes.

Myeloperoxidase activity in peritoneal exsudate

Rats were treated with EEHC (100 mg/kg, p.o.), indomethacin (20 mg/kg, p.o.) or vehicle (distilled water, p.o.). Peritonitis was induced as described before. Samples from the exsudate were collected and then centrifuged with HTAB (14000 rpm, 2 min, 4°C). After that 30 μL from the sample were add to 200 μL of o-dianisidine. Absorbances were then analyzed at the time 0

and 3 min. Myeloperoxidase activity is represented by units of MPO per μL of exsudate.

HE staining and immunohistochemistry analyses for TNF- α

The streptavidin-biotin-peroxidase method was used for immunohistochemistry assays of TNF- α . Three groups of rats were treated with either distilled water (normal controls) or EEHC (100 mg/kg, p.o.). After 30 min, an intraplantar injection of 1% carrageenan was administered to the animals, except the normal controls, and 3 h later, all animals were sacrificed and 5 mm plantar region sections were immersed in buffered formalin solution for 24 h, ethanol and paraffin to histological slides preparation. To analyze TNF- α the sections were then deparaffinized, dehydrated with xylol and ethanol, and immersed in 0.1 M citrate buffer, pH 6, under microwave heating, for 18 min, for antigen recovery. After cooling at room temperature for 20 min, the sections were washed with PBS, followed by a 15-min blockade of endogenous peroxidase with a 3% H₂O₂ solution. The sections were incubated overnight (4°C) with rabbit primary antibody (anti-TNF- α) as 1:200 or 1:400 dilutions in PBS-BSA. On the next day, the sections were washed in PBS and incubated for 30 min with the secondary biotinylated rabbit antibody (anti-IgG) at 1:200 dilution in PBS-BSA. After washing in PBS, the sections were incubated for 30 min with the conjugated streptavidin peroxidase complex (ABC Vectastain® complex, Vector Laboratories, USA). After another washing with PBS, the sections were stained with 3,3'-diaminobenzidine-peroxide (DAB) chromophore, counter-stained with Mayer hematoxylin, dehydrated, and mounted on microscope slides for analysis. Some sections from all groups were used for standard HE staining as well.

Statistical analysis

The results are presented as the mean \pm S.E.M. of 8 animals per group. Statistical analysis was carried out using one way analysis of variance (ANOVA), followed by the Student-Newman-Keuls as the *post hoc* test for multiple comparisons. P-values less than 0.05 ($p < 0.05$) were considered as indicative of statistical significance.

RESULTS

Effect of EEHC on nociception induced by acetic acid in mice

EEHC at the doses of 100, 200 and 400 mg/kg, p.o. reduced the number of abdominal contortions (18.57 ± 4.00 ; 20.89 ± 4.08 ; $23.57 \pm$

5.01 , respectively) when compared to vehicle group (40.66 ± 3.01). The dose of 100 presented the best result (Figure 1).

Effect of EEHC on nociception induced by formalin in mice

EEHC reduced the licking time in both phases of formalin test with all tested doses of 100, 200 and 400 mg/kg, p.o (first phase – 45.63 ± 3.21 ; 52.16 ± 4.40 ; 61.67 ± 7.9 s, respectively) (second phase – 6.42 ± 1.16 ; 32.46 ± 2.21 ; 18.33 ± 4.18 s, respectively) when compared to vehicle (93.72 ± 4.06 ; 94.52 ± 3.78). Indomethacin used as control also reduced the nociception (45.49 ± 2.35 ; 8.76 ± 2.24) (Figure 2).

Effect of EEHC on paw edema induced by carrageenan in rats

In both assays the extract from jatobá reduced the inflammation induced by carrageenan. At the fourth hour EEHC (100 mg/kg, p.o.) reduced both the volume (0.080 ± 0.010 ml) and weigh of edema (90.46 ± 5.31 mg) when compared to the group treated only with vehicle (0.141 ± 0.04 ml and 165.2 ± 2.00 mg). Indomethacin also reduced the parameters (0.043 ± 0.010 ml and 49.37 ± 2.29 mg) (Figures 3 and 4).

Effect of EEHC on paw edema induced by dextran in rats

Extract from jatobá reduced the inflammation induced by dextran. At the second hour EEHC (100 mg/kg, p.o.) reduced the volume (0.045 ± 0.002 ml) of edema when compared to the group treated only with vehicle (0.101 ± 0.009 ml). Ciproheptadin also reduced the parameter (0.048 ± 0.002 ml) (Figure 5).

Effect of EEHC on leukocytes migration induced by carrageenan in rats

EEHC 100 mg/kg, p.o. and indomethacin successfully reduced peritonitis induced by carrageenan in rats (49.17 ± 18.87 and 58.17 ± 9.52 , respectively) in comparison to vehicle (209.80 ± 24.68) (Figure 6).

Effect of EEHC on myeloperoxidase activity in peritoneal exsudate after carrageenan injection in rats

The extract from jatobá at the dose 100 mg/kg, p.o. reduced the activity of MPO (172.20 ± 95.83) on exsudate as well as indomethacin (135.30 ± 87.87). The group treated with vehicle demonstrated intense MPO expression (1350.00 ± 384.00) (Figure 7).

Histopathological changes in the rat paw after EEHC administration in carrageenan model

In Figure 8A is showed the sample of a rat that received only the stimulus of the needle with no carrageenan in it. It could be observed the preservation of tissue architecture with collagen fibers and the presence of fibroblasts. In figure 8B we could see intense edema formation represented by large white spaces and disappearance of collagen fibers. Also carrageenan injection induced leucocytes migration in special neutrophils. In treated groups 8C and 8D it was observed reduction of edema and preservation of tissue. Collagen fibers have suffered only a light destruction after carrageenan application. Neutrophils infiltration was almost completely reduced by EEHC treatment. Fibroblast are present as well in a significantly quantity.

TNF- α expression in the rat paw after EEHC administration in carrageenan model

As it could be noted in figure 9A no staining was showed in the sample that has not received the primary antibody which validates our tests. In figure 9B an intense immunostaining was observed indicating a massive expression of TNF- α in the rats that were injected with 1 % carrageenan. We also may infer that TNF- α plays an important role in this inflammation model. It is possible to observe a strong brown staining in the cytoplasm of neutrophils, eosinophils and macrophages once TNF- α is a well known pro-inflammatory cytokine involved in carrageenan mechanisms. In group treated with EEHC 100 mg/Kg the immunostaining for TNF- α has been markedly reduced, figure 9C. The leukocytes infiltration has also been reduced and even the small quantity of neutrophils that are present has not showed immunostaining. It could be assumed that the Etanolic Extract from *Hymenaea courbaril* at the dose tested reduces the expression of TNF- α in the model of paw edema induced by carrageenan.

DISCUSSION

For the first time a research demonstrates the potential anti-inflammatory and antinociceptive actions from the etanolic extract from *Hymenaea courbaril* L. (EEHC) in animal models. EEHC in all tested doses presented significant reduction on the parameters analyzed. The dose of 100 mg/kg was chosen to be tested in all experiments once this dose has shown to perform the best result in traditional pain and inflammation models. Our data are in accordance with other studies that showed successfully the

effects of plants from the *Leguminosae* family on inflammation and nociception (Oliveira *et al.*, 2009; Gelminia *et al.*, 2012; Cruz *et al.*, 2007). The extract has not showed toxicity by oral administration (data not shown).

The present results indicate that EEHC has a potent anti-inflammatory and antinociceptive activity since it inhibits acetic-acid abdominal writhing, formalin nociception, paw edema induced by both carrageenan and dextran, peritonitis and myeloperoxidase expression also induced by carrageenan. Although these findings are consistent with studies showing these effects of *Leguminosae* in many inflammation models none of them were performed with *Hymenaea courbaril*.

Our results develop a major importance once the bioprospecting potential of *Hymenaea courbaril* are cited as one of the most promising in northeast Brazil specially in the state of Ceará. A recent study has pointed the popular uses of the plant as well as the parts used and the forms of preparation (Cartaxoa *et al.*, 2010). Then preclinical studies as this one add valuable knowledge to further researches.

Carrageenan-induced inflammation in the rat paw represents a model of acute edema formation and hyperalgesia extensively used in the development of nonsteroidal and anti-inflammatory drugs and selective COX-1 and 2 inhibitors. The edema formation is the result of interaction among various inflammatory mediators that increase vascular permeability and/or mediators that increase the blood flow (Sautebin *et al.*, 1995).

The carrageenan-induced edema has been describe as a biphasic event: the early phase, observed around 1 h after the carrageenan injection is related to the production of serotonin, histamine, bradykinin and cyclooxygenase products. The late phase is due to neutrophil infiltration as well as to the continuing production of arachidonic acid metabolites (Salvemini *et al.*, 1996). Our data agree with others obtained in studies using other *Leguminosae* that also orally an extract has reduced the edema extension (Arzumand *et al.*, 2008). EEHC successfully at the dose of 100 mg/kg *per oral* reduced the edema (weight and volume) and the leukocyte migration to peritoneal cavity. The histological analysis confirms the edema reduction and the reduced number of neutrophils that reached the tissue (paw) after carrageenan injection.

Abdominal writhing induced by acetic acid involves the process or release of arachidonic acid metabolites via cyclooxygenase (COX), and prostaglandin biosynthesis (Melo *et al.*, 2008). In this context our results suggest a possible

blockage of prostaglandins production once the models involving carrageenan also are dependent on prostanoids formation.

The formalin test is composed of two distinct phases which differ in chemical mediation and mechanism modulation. The first one is associated to neurogenic origins and the second one is related to inflammation itself (Shibata *et al.*, 1989). EEHC at all tested doses reduced the time of paw linking in both phases. We could infer that besides prostaglandin reduction the extract could also exhibits a central action. Nevertheless, specific experiments must be performed to confirm this assumption.

Furthermore we performed and MPO test in exsudate after peritoneal injection of carrageenan to rats which confirms that EEHC exerts a potent anti-inflammatory action. This extract significantly inhibited MPO release. The result is probably related to reduction of pro-inflammatory mediators and adhesive molecules commonly associated to this model (Scognamillo-Szabó *et al.*, 2005). It must be marked that in the same experiment EEHC reached a result similar to indomethacin, a well-known nonsteroidal drug.

Paw edema induced by dextran is already satisfactory described and involves vascular modification and histamine and serotonin release by mastocytes (Benditt *et al.* 1956). It could be observed that the extract reduced the edema at the second hour and this result also could be related to reduction of these preformatted mediators. Specific models using histamine and serotonin must be developed to evaluate their participation.

The extract from *Hymenaea courbaril* might exerts its anti-inflammatory and

antinociceptive effects through COX-2 inhibition or through more specific mechanisms once EEHC reduced TNF- α expression in cells after carrageenan induced paw edema. It could be question if the mechanism of this enzyme reduction is gene related once drugs that inhibit TNF- α also reduced inflammation and NF- κ B activation (Ozes *et al.*, 1999). Nevertheless, this is yet just an assumption and a motive to further studies.

Procyanidins, terpenoid-derived compounds, demonstrated many bioactive actions such as free radical scavenging (Takahata *et al.*, 2001), inhibition of nitric oxide synthase (Stevens *et al.*, 2002) and secretion of anti-inflammatory interleukin-5 (Mao *et al.*, 2006). The presence of great quantity of this substance in our extract could justify the potent action on the models tested. Following our research isolated components from EEHC will be challenged.

CONCLUSIONS

The sum of all experiments performed confirms the effects of the etanolic extract of *Hymenaea courbaril* on inflammation and nociception for the first time in animal models. The popular uses of this plant are well based although more research must be designed to evaluate its mechanisms of action.

ACKNOWLEDGEMENTS

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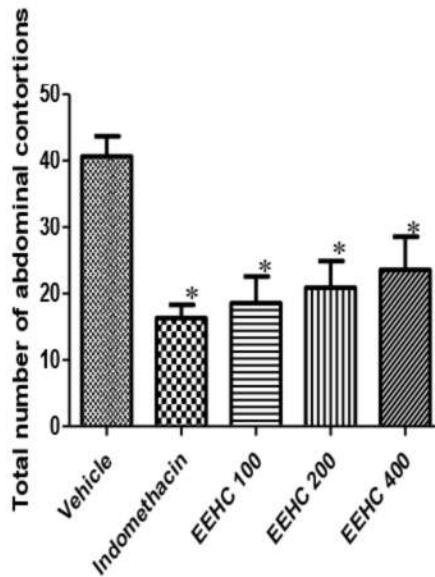


FIGURE 1. Effect of EEHC and indomethacin treatment on abdominal contortions induced by acetic acid in mice. Data are presented as mean \pm S.E.M. from 8 animals. * $p < 0.05$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

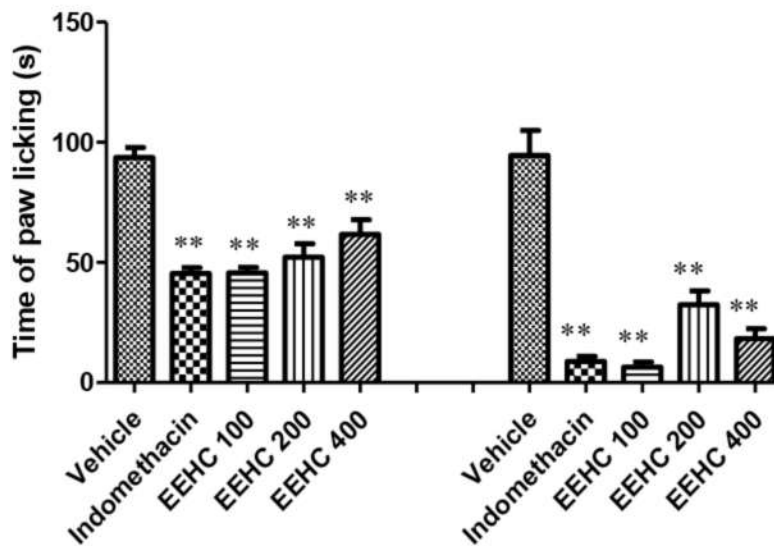


FIGURE 2. Effect of EEHC and indomethacin treatment on nociception induced by formalin in mice. Data are presented as mean \pm S.E.M. from 8 animals. ** $p < 0.01$; *** $p < 0.001$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

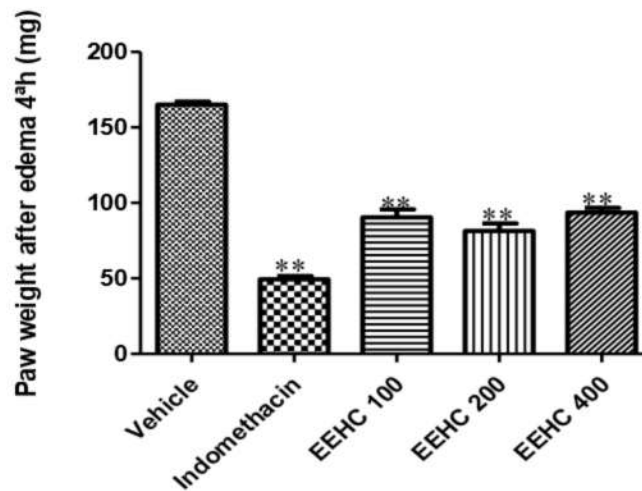


FIGURE 3. Effect of EEHC and indomethacin treatment on paw edema induced by carrageenan in mice measured by weight at the 4^a hour after inflammation induction. Data are presented as mean \pm S.E.M. from 8 animals. *** $p < 0.001$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

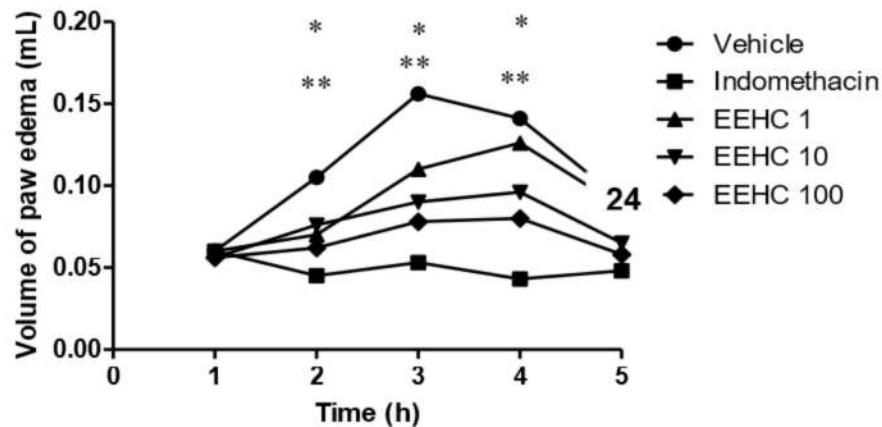


FIGURE 4. Effect of EEHC and indomethacin treatment on paw edema induced by carrageenan in rats. Data are presented as mean \pm S.E.M. from 8 animals. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

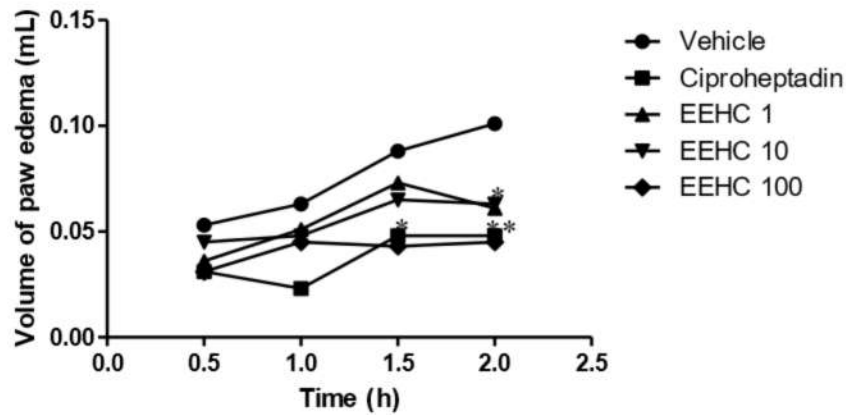


FIGURE 5. Effect of EEHC and indomethacin treatment on paw edema induced by dextran in rats. Data are presented as mean \pm S.E.M. from 8 animals. * $p < 0.05$; ** $p < 0.01$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

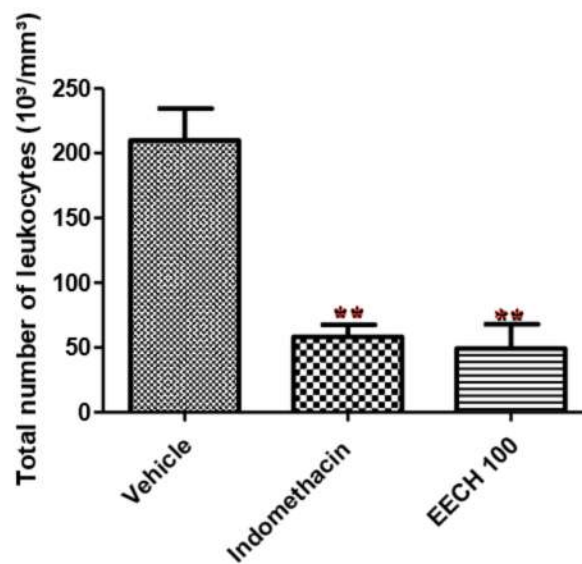


FIGURE 6. Effect of EEHC and indomethacin treatment on leukocyte migration induced by carrageenan in rats. Data are presented as mean \pm S.E.M. from 8 animals. *** $p < 0.001$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

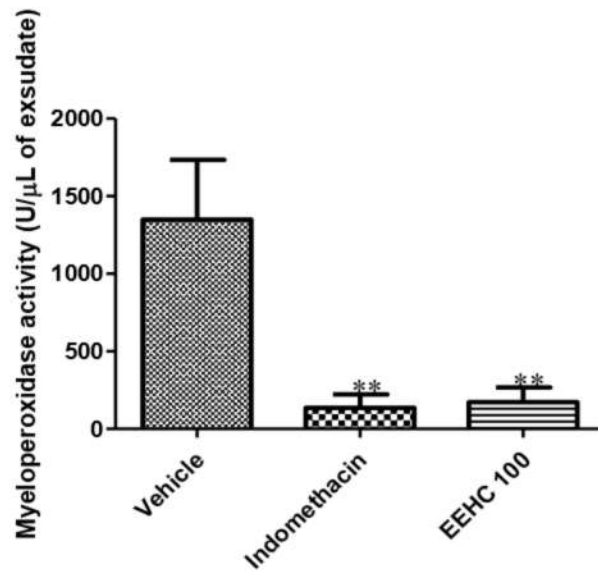


FIGURE 7. Effect of EEHC and indomethacin treatment on myeloperoxidase activity in peritoneal exsudate after carrageenan injection in rats. Data are presented as mean \pm S.E.M. from 8 animals. ** $p < 0.01$ vs. control (vehicle). (ANOVA followed by Student Newman-Keuls test).

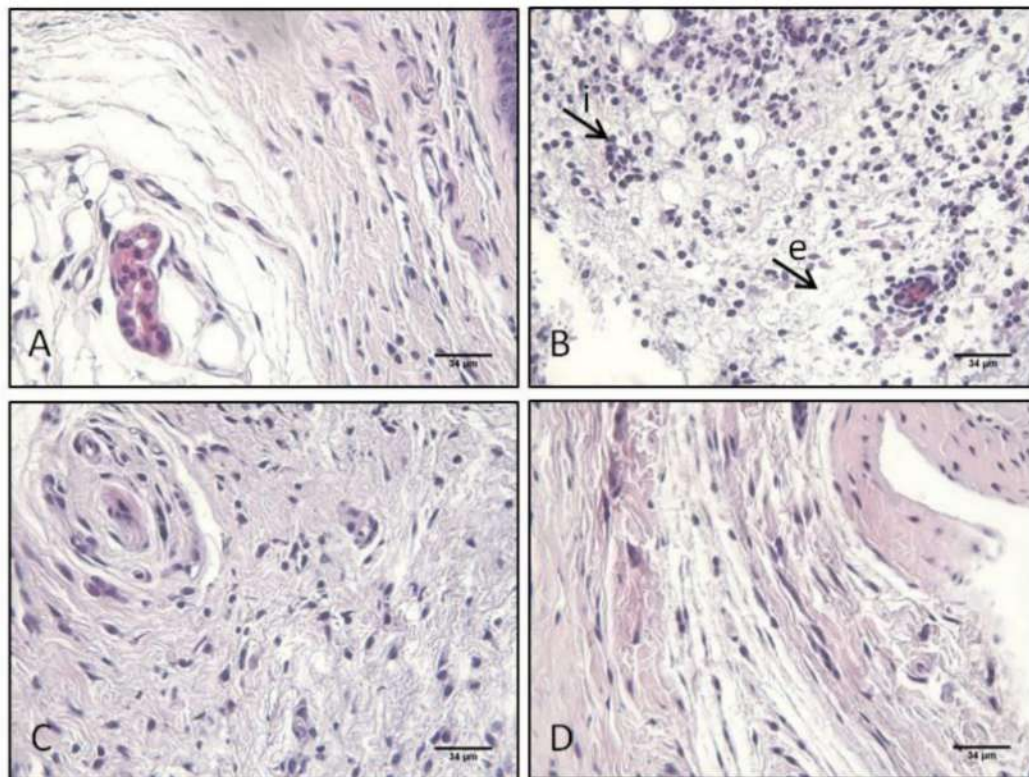


FIGURE 8. Hematoxylin and eosin staining in model of acute paw edema induced by carrageenan in rats. **A.** Shamed control. **B.** Inflammation control. **C.** Indomethacin 20 mg/Kg. **D.** EEHC 100 mg/Kg. e (edema); i (inflammatory cells infiltration); f (collagen fibers preservation) (400x).

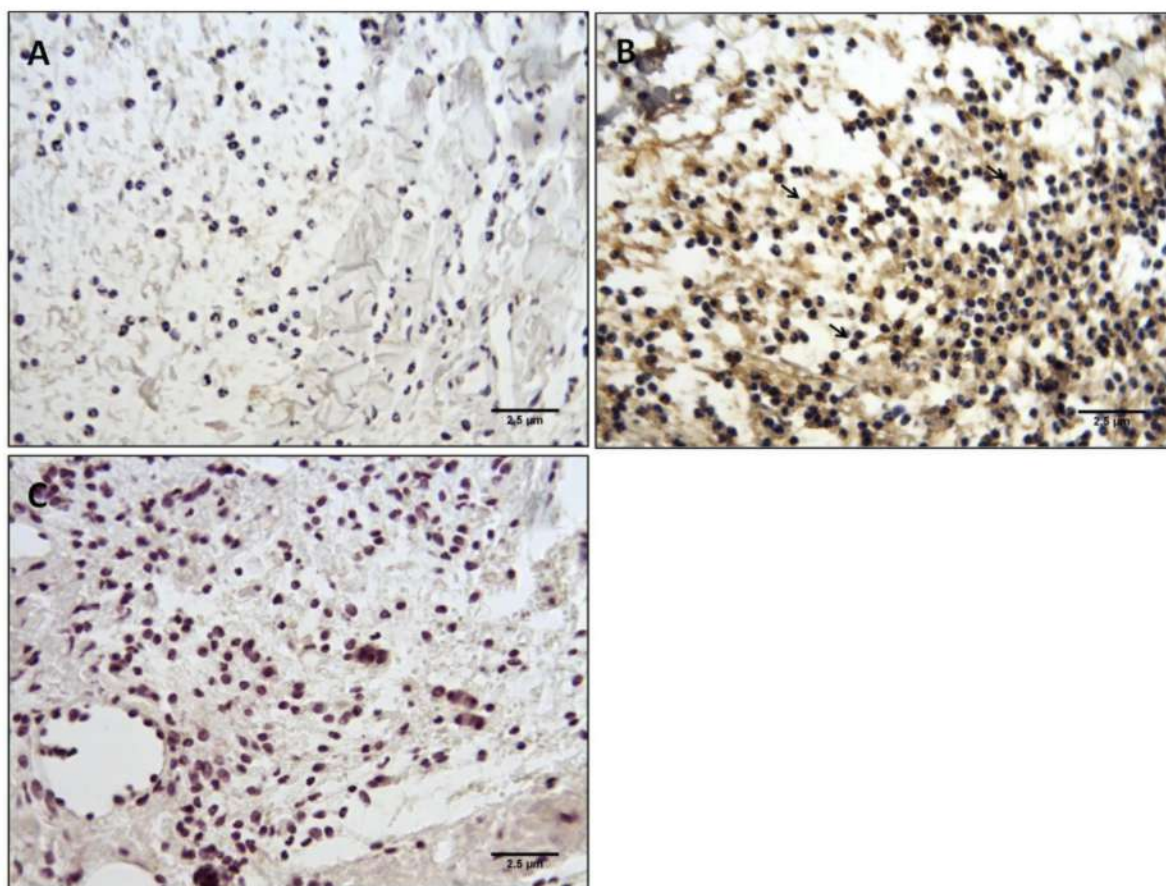


FIGURE 9. Immunohistochemistry for TNF- α in model of acute paw edema induced by carrageenan in rats. **A.** Negative control. **B.** Inflammation control (TNF- α). **C.** EEHC 100 mg/Kg. The brown staining indicates interaction of primary and secondary antibodies and as a consequence the presence of TNF- α . Black arrows indicates polymorphonuclear cell and macrophages stained to TNF- α (400x).

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